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(54) Title: METHODS AND COMPOSITIONS FOR USE IN POTENTIATING ANTIGEN PRESENTATION BY ANTIGEN PRESENTING CELLS

(57) Abstract

The invention provides methods to enhance the antigen–presentation capabilities of cells capable of antigen presentation by contacting the cells with an immunostimulatory oligonucleotide. Through such contact, treated antigen presenting cells are induced to take up antigen (through upregulation of Fc R expression), present antigen (through upregulation of MHC Class I and II as well as CD1d expression), produce co–stimulatory factors (B7 and CD40), provide cell–to–cell adhesion (through upregulation of ICAM expression) and increase Th1 stimulatory cytokine production (e.g., IL–12), all at levels greater than are achieved through contact of the antigen presenting cells with antigen alone. Methods for use of the enhanced antigen presenting cells to activate T cells to respond to antigen are also provided, as are compositions of antigen presenting cells with activity enhanced according to the invention.

METHODS AND COMPOSITIONS FOR USE IN POTENTIATING ANTIGEN PRESENTATION BY ANTIGEN PRESENTING CELLS

STATEMENT OF GOVERNMENT RIGHTS

This invention was made with Government support under Grant No. PO1 AI40682, awarded by the National Institutes of Health. The Government may have certain rights in this invention.

FIELD OF THE INVENTION

The invention relates to methods and oligonucleotide compositions for use in enhancing the antigen-presentation activity of cells which have antigen-presentation capabilities.

HISTORY OF THE RELATED ART

Cognate interactions between antigen-presenting cells (APCs) and lymphocytes play a crucial role in the primary activation of T cells to respond to antigen. These interactions are coordinated by upregulation of MHC, adhesion and co-stimulatory molecules on the surface of antigen-stimulated cells with antigen-presentation capabilities (e.g., dendritic cells, macrophages, B cells and tumor cells), as well as by contact with cytokines. Without the contribution of these varying factors, T cells can respond to antigen stimulation (especially by weakly immunogenic antigens) by developing tolerance rather than reactivity.

For example, tumor antigen vaccine administration can lead to T cell tolerance, thus minimizing the efficacy of the vaccine in countering tumor development. Such tolerance is believed to be the result, in part, of the absence of co-stimulatory signals (e.g., binding by T cells of B7 ligand on APCs). To avoid such tolerance induction, B7 transduced tumor cells are being studied for use as anti-cancer vaccines, with mixed success.

SUMMARY OF THE INVENTION

The invention provides methods for upregulating the expression of certain cell surface molecules on cells capable of antigen-presentation (e.g., B cells and macrophages; collectively, "APCs"), thereby enhancing the antigen-presenting activity of such cells, as compared to levels of expression observed in unstimulated and antigen-stimulated cells. The enhancement of antigen-presenting activity in such cells ("APC-activation" of the cells) is provided by contacting the cells with an immunostimulatory oligonucleotide (ISS) and can be achieved not only in cells pre-primed with ISS before contact with antigen, but also in cells contacted with ISS and antigen in combination. APC-activation of target cells in

Figure 2: *In vivo induction of cell surface molecules on mouse splenic B cells.*
2A and 2B. BALB/c mice were injected i.p. with ISS-ODN (50 μg/ml), M-ODN (50 μg/ml), or saline. Mice were sacrificed on days 2, 7, and 21 after injection, the spleens were harvested, and the cells were stained for expression of B220 (PE) and the activation markers (FITC) for FACS analysis. The histograms represent the FITC staining of gated PE^+ B cells. The numbers in the upper left represent the mean fluorescence, which was used to compare expression levels per cell for the markers that are expressed constitutively in >95% of the cells. Data are representative of the results obtained from 3 different mice per group.

Figure 3: *Inhibition of B cell proliferation does not effect B cell surface molecule expression.* To prevent proliferative response, spleen cells were gamma irradiated (1500 rad) or MMC-treated (50μg/ml) and then incubated with ISS-ODN (1 μg/ml) plus BrdU (15 μg/ml). After 48 hours incubation, cells were stained for B220 (PE) and the activation markers (FITC) checked by FACS analysis. The histograms represent the FITC staining of gated PE^+ B cells. "Untr" represents untreated cells, MMC represents mitomycin-treated cells and "Irrad" represents irradiated cells. The numbers on the right side of the histogram represent the percentage of positive cells in comparison to the media control. The numbers in the upper left represent the mean fluorescence, which was used to compare expression levels per cell for the markers that are expressed constitutively in >95% of the cells. Data are represent values from 3 independent experiments.

Figure 4: *In vitro pUC19 activation of splenic B cell surface molecule expression.*
Spleen cells ($2x10^6$/well) were transfected (using DOTAP) with pUC19 (10 μg/ml) for 4 hours, then washed and incubated for additional 48 hours in culture media and then stained for B220 (PE) and activation markers (FITC) for FACS analysis. The histograms represent the FITC staining of gated PE^+ B cells. The numbers on the right side of the histogram represent the percentage of positive cells in comparison to the media control. The numbers in the upper left represent the mean fluorescence, which was used to compare expression levels per cell for the markers that are expressed constitutively in >95% of the cells. Data represent values from 3 different experiments.

Figure 5: *Bone marrow derived macrophage (BMDM) cytokine and surface molecule expression profiles induced by ISS.*
A. *Cytokine profile obtained from BMDM.* BMDM ($2x10^5$/ml) were cultured *in vitro* with ISS-ODN (1μg/ml), M-ODN (1 μg/ml), pUC19 (10μg/ml), LPS (5 μg/ml), or media alone. The supernatant

Figure 8: *Postulated mechanisms for the induction of a Th1 response by ISS-ODN or ISS enriched pDNA.*

A. ISS-ODN or ISS-pDNA trigger innate immunity to release a distinctive set of Th1 promoting cytokines (IFNs, IL-12, and IL-18) and up-regulate the expression of a distinctive profile of cell surface molecules on APCs (Fc receptors, MHC molecules, co-stimulatory molecules, adhesion molecules and cytokine receptors). These two responses are partially related (dotted lines).

B. Upon antigen encounter, the activated APCs interact with naive Th cells. This cognate interaction between ISS-activated APCs and naive Th cells in the ISS-induces cytokine milieu (i.e., IFNs, IL-12 and IL-18) biases the differentiation of naive Th cells toward a Th1 phenotype.

DETAILED DESCRIPTION OF THE INVENTION

1. *Activity of ISS-ODN APC Activators*

Cancer vaccines have been thought to have promise because, unlike most protective vaccines, they are therapeutic; i.e., they elicit immune responses against antigens (tumor antigens) in residence in the host on target cells. In cancer vaccination, a primary goal is to introduce a tumor antigen (one present on the target tumor cells) to cells with the capability to present antigen to T cells to induce T cell reactivity to the antigen. In all known pathways of primary T cell activation, the efficacy of activation is intimately tied to the density of peptide/MHC complexes displayed on APCs and the particular costimulatory molecules expressed by APCs. Problematically, however, peptide antigens recognized by T cell epitopes tend to be inefficiently presented, leading to T cells of relatively low antigen reactivity.

Thus, potentiation of APC-activation of cells to enhance their capacity to present antigen to T cells is directly correlative with an improvement in T cell reactivity to the presented antigen. This aspect of the invention has significant consequences for the efficacy of T cell-mediated immunotherapies, such as is practiced through use of anti-tumor vaccines.

Those of ordinary skill in the onocological art will be familiar with, or can readily ascertain, materials and methods for use in the construction of anti-tumor vaccines. According to the invention, an ISS-ODN is administered together with the anti-tumor vaccine to serve as an APC activator. The ISS-ODN may be co-administered with the vaccine, administered before vaccination or conjugated to a component of the vaccine (e.g., to the peptide tumor antigen).

involved in antigen uptake (FcγR), antigen presentation (MHC class I, class II, and CD1d), costimulation (B7 and CD40), and cell-cell adhesion (ICAM-1) combined with cytokine production (TNF, IFNs and IL-12), provide the basis of the strong Th1 adjuvant properties and for the pre-priming effect observed for ISS-ODN (see, outline of proposed mechanism of action of ISS-ODN as APC activators shown in Figure 8A and B). These activities would be expected to suppress pathogen replication, enhance pathogen-derived antigen presentation, activate humoral and cell mediated specific immunity, and instruct the adaptive immune system to generate a Th1 response against an invading microbe.

In particular, the uptake of antigen is enhanced by ISS-ODN mediated up-regulation of the Fcγ receptors (CD16/32) while antigen presentation is enhanced by the up-regulation of classical MHC class I and class II molecules (Figures 1-2), as well as non-classical MHC molecules (CD1d; *in vivo*, Figure 2).

ISS-ODN also reduce the threshold number of cognate (cell-to-cell) interactions required for APC activation of lymphocytes by selectively enhancing the expression of ICAM-1 adhesion molecules (Figure 1-2). ICAM-1 binding to its ligand LFA-1 stabilizes interactions between APCs and lymphocytes. Furthermore, ISS-ODN up-regulate the expression of the costimulatory molecules B7 and CD40 on B cells and macrophages. These molecules play a major role in T cell priming, activation, and differentiation via interactions with their specific receptors, CD28 and CD40L, respectively, on naive T cells.

The up-regulation of cell surface molecules on B cells was observed for both splenic B cells (mainly memory cells, Figure 1) and peripheral blood B cells (mainly naive cells). The same pattern of expression was also observed for both *in vitro* (Figure 1) and *in vivo* (Figure 2) ISS-ODN stimulation. The levels of expression *in vivo* were maximal one week after i.p. injection of ISS-ODN and dropped back to the baseline levels after 21 days for most of the parameters evaluated, except for MHC class I, MHC class II, ICAM-1, and CD1d which displayed a sustained expression (Figure 2).

Gamma irradiation of B cells or their treatment with mitomycin C did not modify the expression of the various cell surface molecules described earlier, ruling out the known mitogenic properties of ISS-ODN on B cells as the cause for this differential expression. Neutralizing the cytokines induced by ISS-ODN stimulation resulted only in partial inhibition of the differential expression of some of the cell surface molecules on B cells, indicating their partial role in the observed expression profile.

reported nucleotide sequences of known ISS-ODN. For ease of reference in this regard, the following sources are especially helpful:

Yamamoto, *et al., Microbiol.Immunol.*, 36:983 (1992)
Ballas, *et al., J.Immunol.*, 157:1840 (1996)
Klinman, *et al., J.Immunol.*, 158:3635 (1997)
Sato, *et al., Science*, 273:352 (1996)

Each of these articles are incorporated herein by reference for the purpose of illustrating the level of knowledge in the art concerning the nucleotide composition of ISS-ODN.

In particular, ISS-ODN useful in the invention include those which have the following hexameric nucleotide sequences:

1. ISS-ODN having "CpG" dinucleotides; and,
2. Inosine and/or uracil substitutions for nucleotides in the foregoing hexamer sequences for use as RNA ISS-ODN.

For example, DNA based ISS-ODN useful in the invention include those which have the following hexameric nucleotide sequences:

AACGTT, AGCGTC, GACGTT, GGCGTT, AACGTC, AGCGTC, GACGTC, GGCGTC, AACGCC, AGCGCC, GACGCC, GGCGCC, AGCGCT, GACGCT, GGCGCT, TTCGAA, GGCGTT and AACGCC (respectively, SEQ.ID.Nos. 1-18).

ISS-ODN may be single-stranded or double-stranded DNA, single or double-stranded RNA and/or oligonucleosides. The ISS-ODN may or may not include palindromic regions. If present, a palindrome may extend only to a CpG motif, if present, in the core hexamer sequence, or may encompass more of the hexamer sequence as well as flanking nucleotide sequences.

The nucleotide bases of the ISS-ODN which flank the CpG motif of the core hexamer and/or make up the flanking nucleotide sequences may be any known naturally occurring bases or synthetic non-natural bases (e.g., TCAG or, in RNA, UACGI). Oligonucleosides may be incorporated into the internal region and/or termini of the ISS-ODN using conventional techniques for use as attachment

Confirmation that a particular oligonucleotide has the properties of an ISS-ODN useful in the invention can be obtained by evaluating whether the ISS-ODN affects cytokine secretion and IgG antibody isotype production as described in Section A.2(e), above. Details of *in vitro* techniques useful in making such an evaluation are given in the Examples; those of ordinary skill in the art will also know of, or can readily ascertain, other methods for measuring cytokine secretion and antibody production along the parameters taught herein.

For use in the methods of the invention, the ISS-ODN APC activators of the invention will take the form of free ISS-ODN oligonucleotides, ISS-ODN oligonucleotide-peptide conjugates and ISS-containing recombinant expression vectors (data regarding the activity of ISS-ODN conjugates and ISS-ODN vectors are set forth in co-pending, commonly assigned U.S. patent applications Serial Nos. 60/028,118 and 08/593,554; data from which is incorporated herein by reference to demonstrate ISS-ODN immunostimulatory activity *in vivo*). In a vaccine composition, antigen may be co-delivered (separately or in an admixture with free oligonucleotides), expressed recombinantly from a plasmid (especially one containing the ISS-ODN moiety in the backbone) or conjugated to an antigen.

Examples of other useful conjugate partners include any immunogenic antigen (including allergens, live and attenuated viral particles and tumor antigens), targeting peptides (such as receptor ligands, antibodies and antibody fragments, hormones and enzymes), non-peptidic antigens (coupled via a peptide linkage, such as lipids, polysaccharides, glycoproteins, gangliosides and the like) and cytokines (including interleukins, interferons, erythropoietin, tumor necrosis factor and colony stimulating factors). Such conjugate partners can be prepared according to conventional techniques (e.g., peptide synthesis) and many are commercially available.

Those of ordinary skill in the art will also be familiar with, or can readily determine, methods useful in preparing oligonucleotide-peptide conjugates. Conjugation can be accomplished at either termini of the ISS-ODN or at a suitably modified base in an internal position (e.g., a cytosine or uracil). For reference, methods for conjugating oligonucleotides to proteins and to oligosaccharide moieties of Ig are known (see, e.g., O'Shannessy, *et al., J.Applied Biochem.,* 7:347 (1985), the disclosure of which is incorporated herein by reference solely to illustrate the standard level of knowledge in the art concerning oligonucleotide conjugation). Another useful reference is Kessler: "Nonradioactive Labeling Methods for Nucleic Acids", in Kricka (ed.), *Nonisotopic DNA Probe Techniques* (Acad.Press, 1992)).

For review, however, those of ordinary skill may wish to consult Ausubel, *Current Protocols in Molecular Biology*, supra.

Briefly, construction of recombinant expression vectors (including those which do not code for any protein and are used as carriers for ISS-ODN) employs standard ligation techniques. For analysis to confirm correct sequences in vectors constructed, the ligation mixtures may be used to transform a host cell and successful transformants selected by antibiotic resistance where appropriate. Vectors from the transformants are prepared, analyzed by restriction and/or sequenced by, for example, the method of Messing, *et al., (Nucleic Acids Res.*, 9:309, 1981), the method of Maxam, *et al., (Methods in Enzymology*, 65:499, 1980), or other suitable methods which will be known to those skilled in the art. Size separation of cleaved fragments is performed using conventional gel electrophoresis as described, for example, by Maniatis, *et al., (Molecular Cloning*, pp. 133-134, 1982).

Host cells may be transformed with expression vectors and cultured in conventional nutrient media modified as is appropriate for inducing promoters, selecting transformants or amplifying genes. The culture conditions, such as temperature, pH and the like, are those previously used with the host cell selected for expression, and will be apparent to the ordinarily skilled artisan. If a recombinant expression vector is utilized as a carrier for the ISS-ODN of the invention, plasmids and cosmids are particularly preferred for their lack of pathogenicity. However, plasmids and cosmids are subject to degradation *in vivo* more quickly than viruses. Alternatively, viral vectors that can be utilized in the invention include adenovirus, adeno-associated virus, herpes virus, vaccinia or an RNA virus such as a retrovirus. Of the viral vector alternatives, adeno-associated viruses would possess the advantage of low pathogenicity. The relatively low capacity of adeno-associated viruses for insertion of foreign genes would pose no problem in this context due to the relatively small size in which ISS-ODN of the invention can be synthesized.

If modification of the phosphate group of an ISS-ODN is desired (e.g., to increase its bioavailability), the techniques for making phosphate group modifications to oligonucleotides are known in the art and do not require detailed explanation. For review of one such useful technique, the intermediate phosphate triester for the target oligonucleotide product is prepared and oxidized to the naturally occurring phosphate triester with aqueous iodine or with other agents, such as anhydrous amines. The resulting oligonucleotide phosphoramidates can be treated with sulfur to yield phophorothioates. The same general technique (excepting the sulfur treatment step) can be applied to yield methylphosphoamidites from methylphosphonates. For more details concerning phosphate group

the text. All of these references are incorporated herein for the sole purpose of illustrating the level of knowledge and skill in the art concerning drug delivery techniques.

ISS-ODN may also be prepared as part of a drug delivery system, such as a liposome. Liposomes are artificial membrane vesicles which are useful as delivery vehicles *in vitro* and *in vivo*. It has been shown that large unilamellar vesicles (LUV), which range in size from 0.2-4.0 μm can encapsulate a substantial percentage of an aqueous buffer containing large macromolecules. RNA, DNA and intact virions can be encapsulated within the aqueous interior and be delivered to cells in a biologically active form (Fraley, *et al., Trends Biochem. Sci.*, 6:77, 1981). In addition to mammalian cells, liposomes have been used for delivery of polynucleotides in plant, yeast and bacterial cells. In order for a liposome to be an efficient gene transfer vehicle, the following characteristics should be present: (1) encapsulation of the genes encoding the antisense polynucleotides at high efficiency while not compromising their biological activity; (2) preferential and substantial binding to a target cell in comparison to non-target cells; (3) delivery of the aqueous contents of the vesicle to the target cell cytoplasm at high efficiency; and (4) accurate and effective expression of genetic information (Mannino, *et al., Biotechniques*, 6:682, 1988).

The composition of the liposome is usually a combination of phospholipids, particularly high-phase-transition-temperature phospholipids, usually in combination with steroids, especially cholesterol. Other phospholipids or other lipids may also be used. The physical characteristics of liposomes depend on pH, ionic strength, and the presence of divalent cations.

Examples of lipids useful in liposome production include phosphatidyl compounds, such as phosphatidylglycerol, phosphatidylcholine, phosphatidylserine, phosphatidylethanolamine, sphingo-lipids, cerebrosides, and gangliosides. Particularly useful are diacylphosphatidylglycerols, where the lipid moiety contains from 14-18 carbon atoms, particularly from 16-18 carbon atoms, and is saturated. Illustrative phospholipids include egg phosphatidylcholine, dipalmitoylphosphatidylcholine and distearoylphosphatidylcholine.

The targeting of liposomes can be classified based on anatomical and mechanistic factors. Anatomical classification is based on the level of selectivity, for example, organ-specific, cell-specific, and organelle-specific. Mechanistic targeting can be distinguished based upon whether it is passive or active. Passive targeting utilizes the natural tendency of liposomes to distribute to cells of the reticulo-endothelial system (RES) in organs which contain sinusoidal capillaries. Active

suitable pharmaceutical preparation, transdermal transmission, injection and epidermal administration.

For transdermal transmission, absorption promoters or iontophoresis are suitable methods. For review regarding such methods, those of ordinary skill in the art may wish to consult Chien, *supra* at Ch. 7. Iontophoretic transmission may be accomplished using commercially available "patches" which deliver their product continuously via electric pulses through unbroken skin for periods of several days or more. Use of this method allows for controlled transmission of pharmaceutical compositions in relatively great concentrations, permits infusion of combination drugs and allows for contemporaneous use of an absorption promoter.

Ophthalmic administration involves invasive or topical application of a pharmaceutical preparation to the eye. Eye drops, topical cremes and injectable liquids are all examples of suitable mileaus for delivering drugs to the eye.

Systemic administration involves invasive or systemically absorbed topical administration of pharmaceutical preparations. Topical applications as well as intravenous and intramuscular injections are examples of common means for systemic administration of drugs.

E. *Dosing Parameters for ISS-ODN APC Activators*

A particular advantage of the ISS-ODN of the invention is their capacity to exert APC activating activity even at relatively minute dosages. Although the dosage used will vary depending on the clinical goals to be achieved, a suitable dosage range is one which provides up to about 1-1000 μg of ISS-ODN/ml of carrier in a single dosage. In view of the teaching provided by this disclosure, those of ordinary skill in the clinical arts will be familiar with, or can readily ascertain, suitable parameters for administration of ISS-ODN according to the invention.

In this respect, it should be noted that the activity of ISS-ODN in the invention is essentially dose-dependent. Therefore, to increase ISS-ODN potency by a magnitude of two, each single dose is doubled in concentration. Clinically, it may be advisable to administer the ISS-ODN in a low dosage (e.g., about 1 μg/ml to about 50 μg/ml), then increase the dosage as needed to achieve the desired therapeutic goal. Alternatively, a target dosage of ISS-ODN can be considered to be about 1-10 μM in a sample of host blood drawn within the first 24-48 hours after administration of ISS-ODN. Based on current studies, ISS-ODN are believed to have little or no toxicity at these dosage levels.

EXAMPLE II

ISS-ODN induce in vitro up-regulation of a distinctive profile of cell surface markers on splenic B cells

Female BALB/c mice and wild type (wt) control (C57B1/6) mice (six to ten weeks of age) were purchased from The Jackson Laboratories (Bar Harbor, ME). TCR-OVA (DO11.10) transgenic (TG) mice are produced on the BALB/c background to be responsive to a model antigen (ovalbumin, or "OVA") (David Broide, UCSD).

Splenic T cells and APCs were prepared from BALB/c TCR-OVA TG mice. The animals were injected i.d. with saline, ISS-ODN (50 μg/mouse) or M-ODN (50 μg/mouse) on days 0 and 7. On day 14 mice were sacrificed and the spleens were harvested as previosuly described (23) to obtain APCs. To enrich the APCs, splenocytes were treated with anti-CD8 abs (3.155) and anti-CD4 abs (RL172) followed by incubation with guinea pig complement (Pel-Freeze, Rogers, Arkansas) as described (24), and fixed with MMC (50 μg/ml, 30 min. at 37°C) prior to being used as accessory cells. Splenocytes were obtained from naive TCR-OVA TG mice and purified for T cells by negative selection using the following antibody cocktail: J11D (anti-HSA), CA4.12 (anti-Ia), RA36B2 (anti-B220), M5.114 (anti-Ia, isotype IgG2a), MAR.18 (mouse anti-Rat IgG) with guinea pig complement. The resultant T-cell preparations (>95% purity by FACS staining), 10^5/well, were mixed with the same number of accessory cells and hen egg ovalbumin (OVA, 10 μg/ml, grade 5, Sigma) for 3 or 4 days. The cultures were incubated with [^{3}H] thymidine (1 μCi per well) (ICN Pharmaceuticals Inc., Irvine, CA) for 18 hours. Cells were harvested and [^{3}H] thymidine incorporation was determined with a 1450 Microbeta liquid scintillation counter (Wallac, Turku, Finland).

Incubation of murine splenocytes with ISS-ODN for 48 hours resulted in the up-regulation of a distinctive profile of cell surface markers on the B220$^+$ population (Figure 1). As determined by FACS analysis, incubation with ISS clearly enhanced the expression of MHC class I, MHC class II, B7-2, CD40, ICAM-1, CD16/32, IFN-γR and IL-2R. A slight up-regulation was observed for B7-1. In contrast, CD23 expression was down-regulated, while no differences in the expression of CD49b, CD1d (Figure 1), CD49a, CD49c-f, IL-1R, and IL-6 R (data not shown) were observed in the ISS-ODN vs. Media or M-ODN treated cells. The cell surface profile induced by ISS stimulation of purified B splenocytes was similar to that observed for B cells (B220$^+$) from ISS stimulated splenocytes.

macrophage-like morphology. Cells then were washed and re-cultured with media containing ISS-ODN (1 μg/ml), M-ODN (1 μg/ml), LPS (5 μg/ml), pUC19 (10 μg/ml) or methyl-pUC19 (10 μg/ml). After 48 hours the cells were harvested by incubation with ice-cold PBS and prepared for either FACS analysis or a cytokine release assay.

After 8-10 days in culture, bone marrow derived macrophages (BMDM) were washed extensively and incubated with ISS-ODN, M-ODN, pUC19, methyl-pUC19 or LPS for 48 hours. Cytokine production was assessed by ELISA and the cell surface marker expression profile was analyzed by FACS. As shown in Figure 5A, ISS-ODN an dpUC19 but not M-ODN or methyl-pUC19 induced the production of IL-6 and IL-12 (IFN-γ was not induced and TNF-α was not detected at 48 hours). As shown in Figure 5B, expression of MHC class I, B7-1, CD40, CD16/32 and ICAM-1 on BMDM was also enhanced. In contrast to B cells, ISS-ODN or pUC19 did not modify the expression of MHC class II, B7-2, IL-2R or CD23 on BMDM, probably due to the lack of IFN-γ induction in this system.

Interestingly, based on the cytokine levels measured in the supernatants, BMDM derived from BALB/c mice secreted 17 fold more IL-6 and 43 fold more IL-12 than did BALB/c splenocytes upon stimulation with ISS-ODN, suggesting that these cells play a major role in the generation of the cytokine milieu induced by ISS-ODN-based DNAs *in vivo*.

EXAMPLE V

ISS-ODN enhancement of the functional ability of APCs to initiate an immune response in vivo

The data presented in the preceding examples demonstrate that, in addition to cytokine production and B cell proliferation, ISS-ODN up-regulates a distinct profile of cell surface molecules on various APCs (e.g., macrophages and B cells) involved in priming and shaping the subsequent T cell response (i.e., Th1 vs. Th2). Two different *in vivo* systems were used to evaluate the functionality of ISS-treated APCs (splenocytes) to activate T cells.

First, TCR-OVA TG mice were injected with ISS-ODN, M-ODN or saline. TCR-OVA TG mice with naive T cells (2 mice/group) were injected intradermally (i.d.) At the base of the tail with ISS-ODN (50 μg/mouse), M-ODN (50 μg/mouse), or saline on days 0 and 7. Mice were killed at day 14 for T cell proliferation and cytokine release assays. Twelve weeks after protein injection the mice were sacrificed. In the second set of experiments, BALB/c mice (4 mice/group) were injected i.d. (at the base of the tail) with ISS-ODN (50 μg/mouse) at the same site. As control groups, mice were co-injected i.d. at day 0 with either ISS-ODN (50 μg/mouse) or M-ODN (50 μg/mouse) plus ß-gal (10

To analyze the effect of pDNA on the cell surface profile, mouse splenocytes were incubated or transfected with pUC19 for 48 hours prior to antibody staining and analysis by FACS. More particularly, transfections of mouse splenocytes with pUC19 (10 μg/ml) and methyl-pUC19 (10 μg/ml) were performed with DOTAP (Boehringer Manheim). Mouse spleen cells were resuspended at $3x10^5$ cells/ml in teflon tubes and incubated with a mix of plasmid and DOTAP for 4 hours at 37 C, and 5% CO_2. Cell were washed twice and resuspended at $2x10^6$ cells/ml in culture media. After 48 hours of incubation, the supernatants were collected for cytokine ELISA and the cells harvested and stained for FACS analysis as described above. Additionally, FACS analysis of splenocytes incubated with pUC19 (10μg/ml) and methyl-pUC19 (10 μg/ml) was made under the same conditions mentioned above without DOTAP.

As shown in Figure 4, transfection with pUC19 induced a similar cell surface expression profile on B splenocytes as did incubation with ISS-ODN (Figure 1) while incubation with pUC19 resulted in a weak to marginal induction of cell surface molecules on these cells.

EXAMPLE VII

Induction of cell surface molecules on B cells is not dependent upon B cell proliferation

To evaluate whether the effect of ISS-ODN on the expression of surface molecules on B cells was related to its mitogenic effect on B cells, splenocytes were gamma irradiated or treated with MMC and then incubated with ISS-ODN or M-ODN. The expression of surface markers was analyzed 48 hours later. The inhibition of proliferation was confirmed by the lack of BrdU incorporation.

More particularly, to inhibit the mitogenic effect of ISS-ODN (11,12), murine spleen cells were gamma irradiated (1500 rad) or treated with MMC (50 μg/ml for 30 min. At 37°C). To check whether under these conditions ISS-ODNs still modify the expression of surface molecules on B cells, splenocytes were incubated with ISS-ODN (1 μg/ml), M-ODN (1 μg/ml), LPS (5 μg/ml) or media alone. After a 48 hour incubation, cells were stained for expression of B220 versus cell surface markers. Additionally, the cells were incubated with BrdU (15 μg/ml, Boehringer Mannheim, Indianapolis, IN) in order to check the inhibition of proliferation after the different treatments.

As shown in Figure 3, ISS-ODN up-regulates the expressioin of cell surface molecules despite the inhibition of the B cell proliferative response of ISS either by irradiation or by MMC treatment,

The invention claimed is:

1. A method for inducing activation of T cells to respond to an antigen, the method comprising contacting cells which are capable of antigen-presentation with an immunostimulatory oligonucleotide (ISS-ODN) to produce antigen presenting cells (APCs) with enhanced antigen-presentation capabilities, as compared to antigen-activated APCs; which APCs with enhanced antigen-presentation capabilities then present antigen to the T cells.

2. The method according to Claim 1 wherein the APCs with enhanced antigen-presenting capabilities are B cells.

3. The method according to Claim 1 wherein the APCs with enhanced antigen-presenting capabilities are bone-marrow derived macrophages.

4. The method according to Claim 1 wherein the APCs with enhanced antigen-presenting capabilities are tumor cells.

5. The method according to Claim 4 wherein the antigen is a tumor antigen.

6. The method according to Claim 1 wherein the APCs with enhanced antigen-presentation capabilities are produced *in vivo* by administering the ISS-ODN to a host.

7. The method according to Claim 1 wherein the APCs which have enhanced antigen-presentation capabilities are produced *in vitro*.

8. The method according to Claim 6 wherein the ISS-ODN is administered to the host concomittantly with the antigen.

9. The method according to Claim 6 wherein the ISS-ODN is administered to the host without the antigen.

10. The method according to Claim 7 wherein the T cells are activated through *in vitro* contact with the APCs which have enhanced antigen-presentation capabilities.

FIGURE 1A

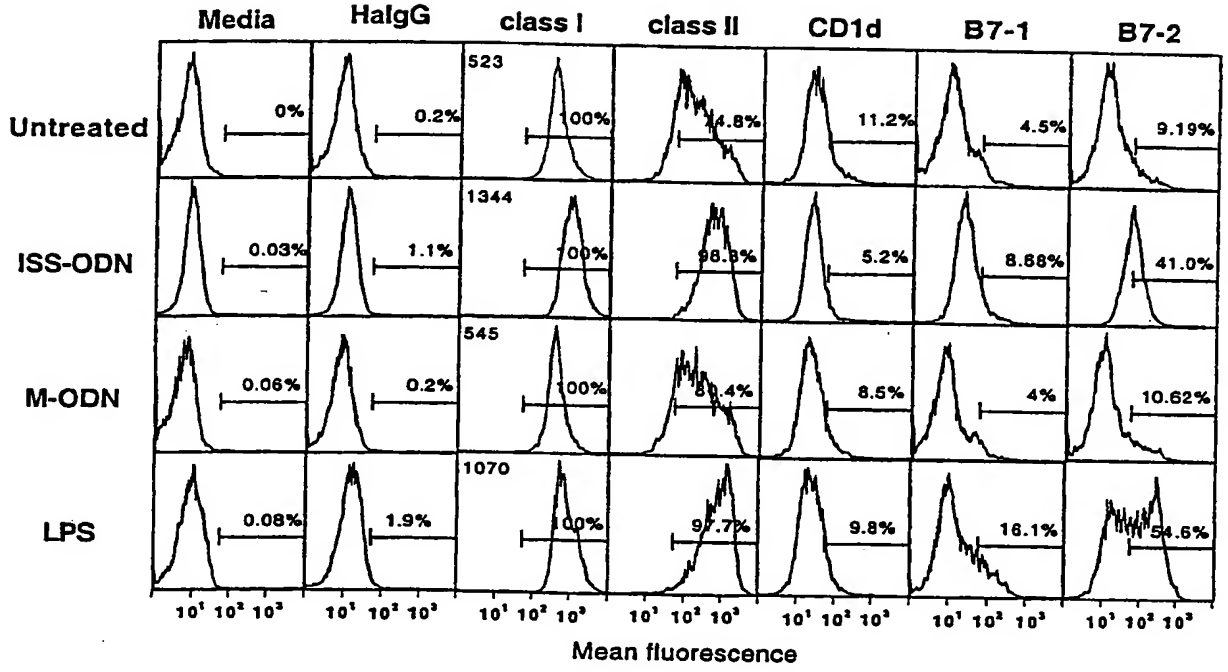
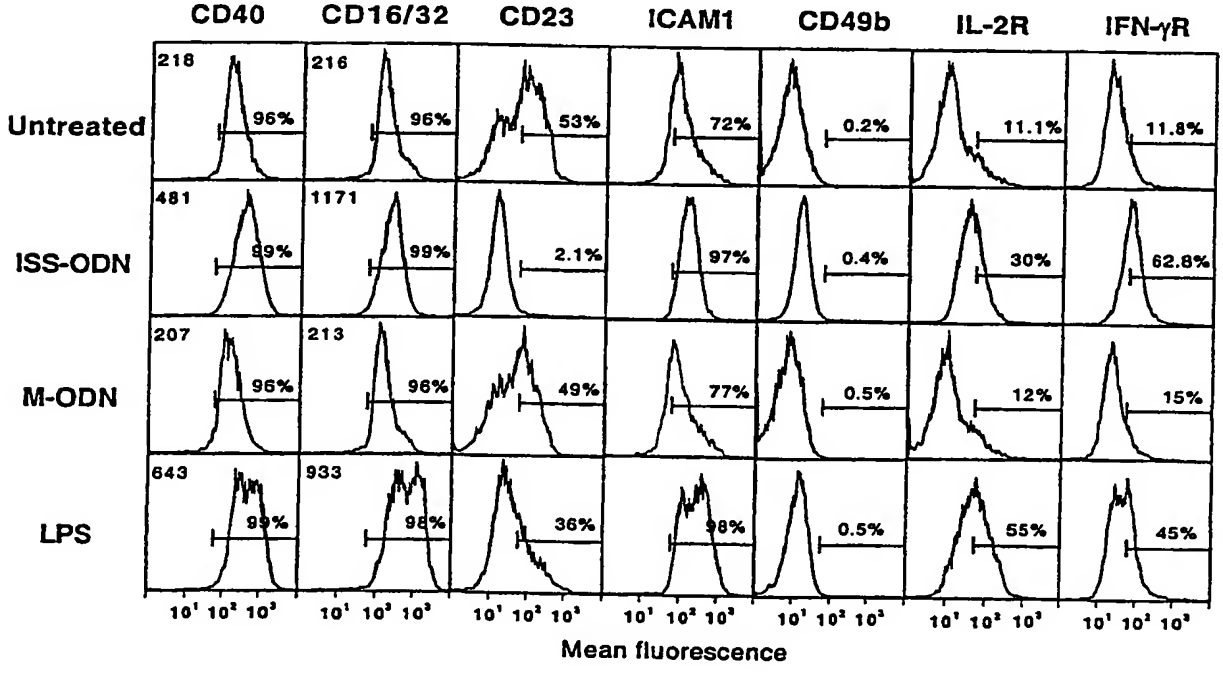


FIGURE 1B



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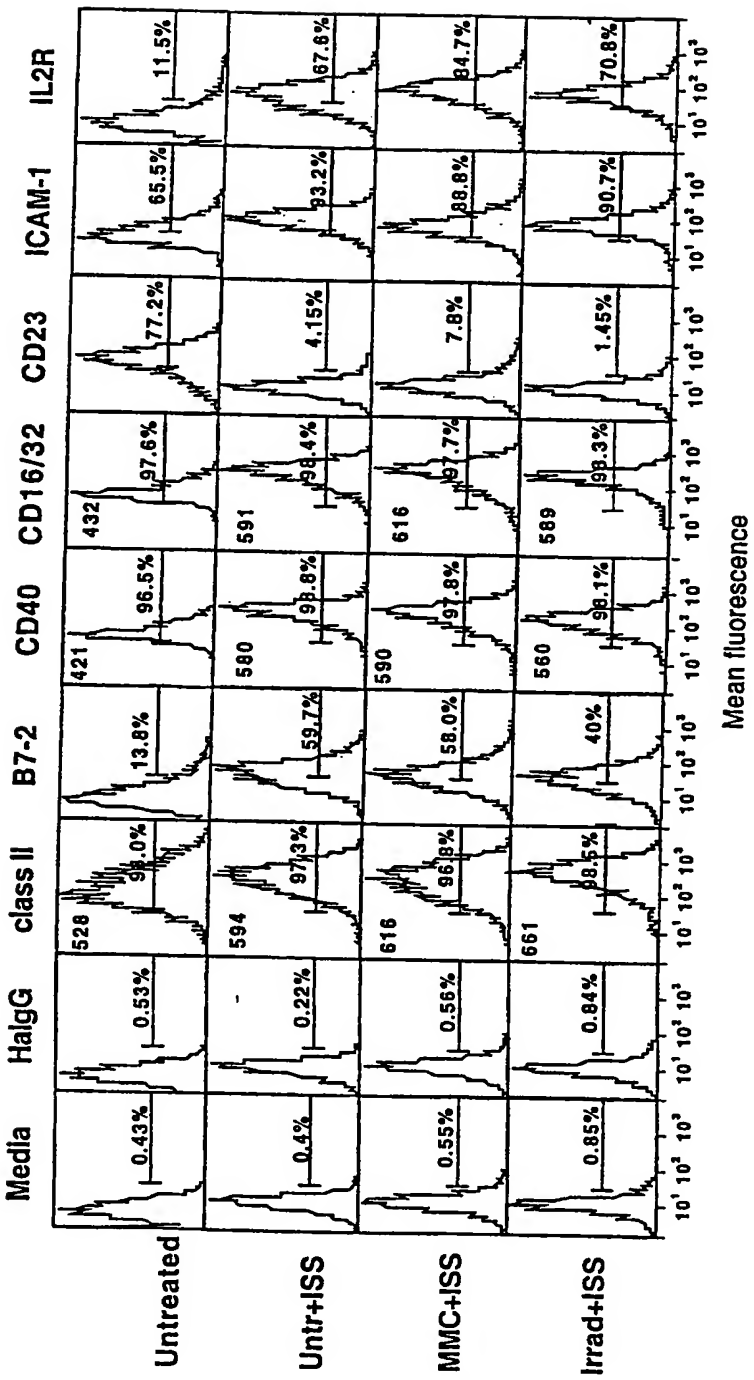
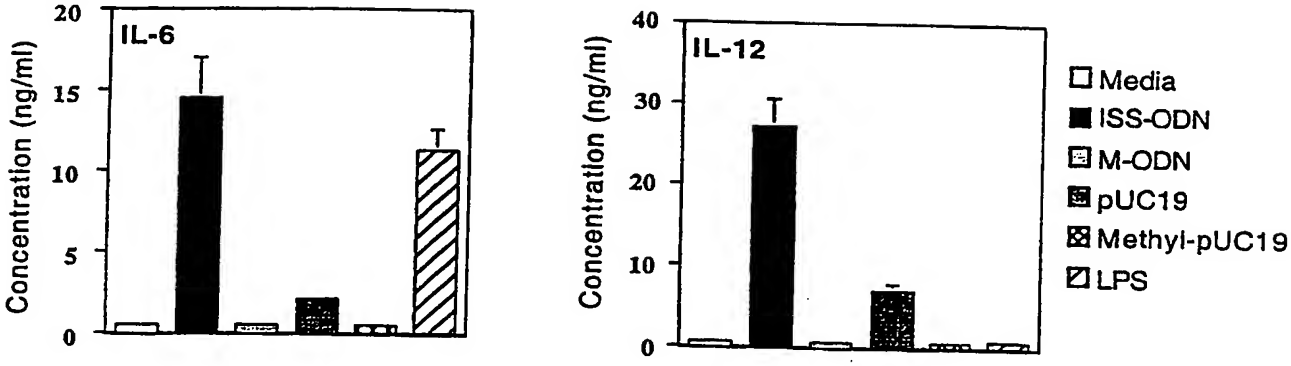


FIGURE 5A



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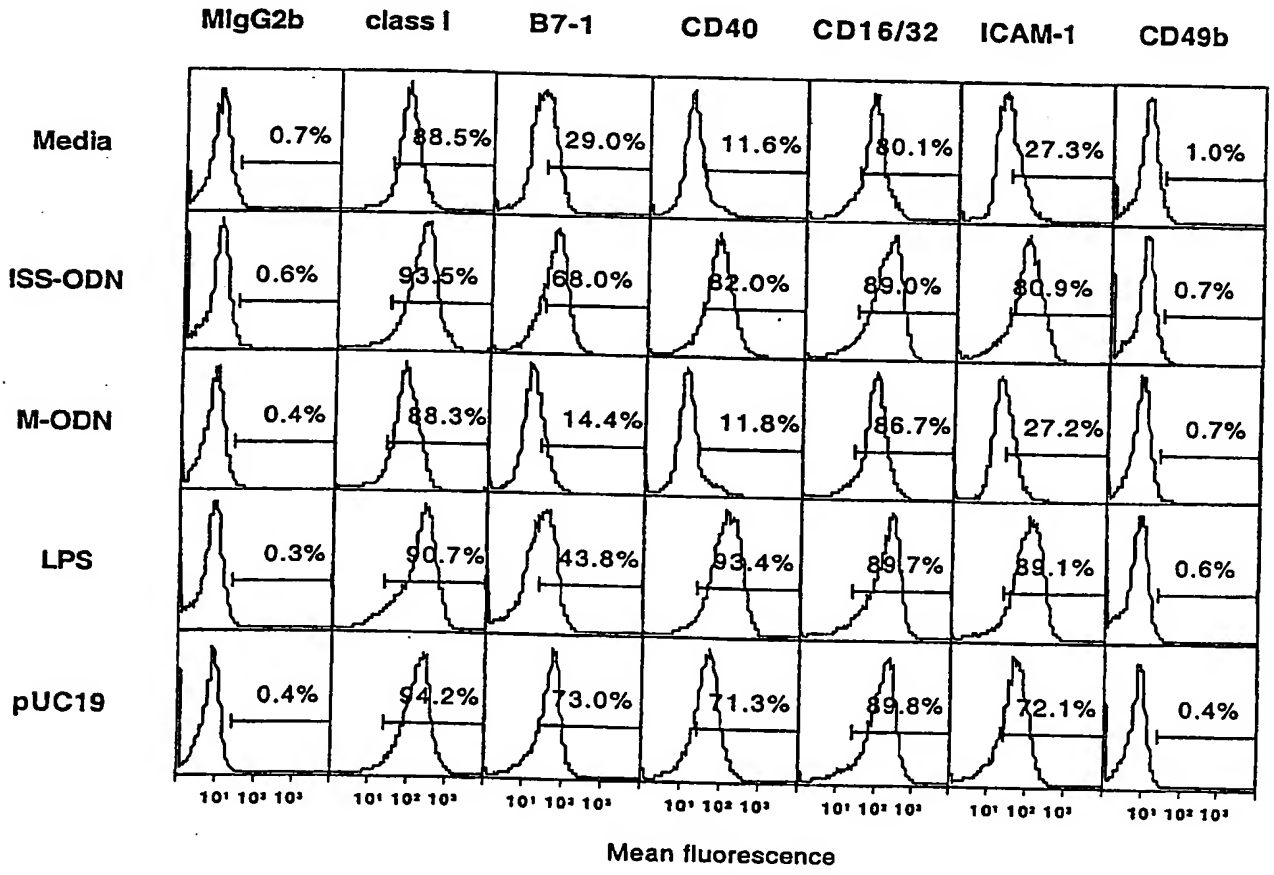


FIGURE 7A
IFN-γ Production

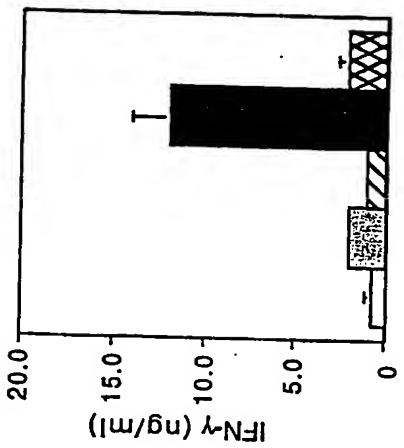
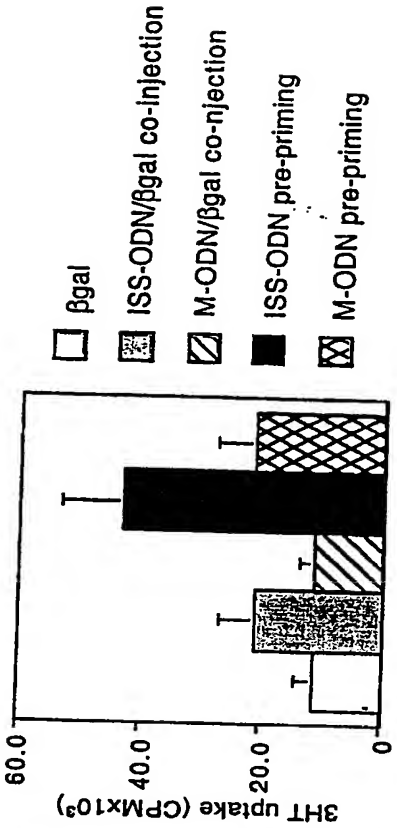


FIGURE 7B
T cell proliferation



SEQUENCE LISTING

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/09664

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) : A61K 35/00, 48/00; C12N 15/63, 15/85, 15/86; A01N 1/02
US CL :514/44; 435/320.1, 325, 1.1; 424/93.1, 93.21

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 514/44; 435/320.1, 325, 1.1; 424/93.1, 93.21

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

NONE

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

NONE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	LIPFORD et al. Immunostimulatory DNA: sequence-dependent production of potentially harmful or useful cytokines. Dec. 1997. Eur. J. Immunol. Vol. 27, pages 3420-3426. See page 3425, column 1, line 2 and page 3422, sentence bridging columns 1 and 2.	1, 6, 12, 14-17, 20 and 21 --- 1-4 and 6-21
Y	JAKOB et al. Activation of cutaneous dendritic cells by CpG-containing oliodeoxynucleotides: a role for dendritic cells in the augmentation of Th1 responses by immunostimulatory DNA. J. Immunol. Vol. 161. pages 30342-3049. See page 3047, column 1, lind 5; 3048, sentence bridging columns 1 and 2.	1-4 and 6-21

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

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Date of the actual completion of the international search

15 MAY 2000

Date of mailing of the international search report

13 JUN 2000

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